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Physiological analysis of salt stress behaviour of citrus species and genera: Low chloride accumulation as an indicator of salt tolerance

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Abstract

Tolerant citrus rootstocks are defined as Cl^- excluders. However, little is known about the salt tolerance of cultivars used as scions, particularly the tolerance of monoembryonic citrus genotypes. To enhance the genetic resources for generating improved hybrid rootstocks, the evaluation of large samples of citrus species, including both monoembryonic and polyembryonic genotypes, is necessary. In this study, 12 citrus genotypes representing the major *Citrus* species and all the three genera of the *Rutaceae* family were subjected to moderate salt stress (75 mM) for 12 weeks to characterise their physiological response to salt stress. Various symptoms and physiological parameters were evaluated to characterise their salt sensitivity. These included plant growth (stem diameter), leaf chlorophyll content, leaf flavonoid content, maximum quantum yield of PSII $[(F_m - F_0)/F_m)]$, net photosynthesis, stomatal conductance and leaf Na^+ and Cl^- contents. The results clearly demonstrated that the most salt sensitive genotypes accumulated high concentrations of Na^+ and Cl^- and maintained a fair growth and photosynthetic rate. By contrast, salt-tolerant genotypes accumulated less Na^+ and Cl^- and decreased their growth and gas exchange. Poncire commun citron and Marumi kumquat were the most sensitive species, while mandarins, pummelo and Australian sour orange were the most tolerant species. Among the genotypes, Engedi pummelo presented a specific trait for salt tolerance that has not been previously reported. Taken together, the results suggest that low leaf chloride content can be used as an indicator of salt stress tolerance in citrus genotypes. Exploitation of this indicator will enable the improved evaluation of citrus genetic resources and should lead to the identification of new sources of tolerance for rootstock breeding.

Keywords: Chloride content; Citrus; Diversity; Rootstock; Salt stress

1. Introduction

Salinity is one of the major abiotic constraints that affects agriculture, especially in countries where irrigation is required (Flowers, 2004). The adverse effects of salinity on plant growth and development are associated with low osmotic potential, accumulation of ions to toxic levels, and nutritional imbalances (Ashraf and Harris, 2004; Byrt and Munns, 2008). This usually leads to a reduction in plant growth and fruit yields (Levy et al., 1979; Maas, 1993; Storey and Walker, 1998). The primary effect of high salt concentration in plants is stomatal closure. This causes a low transpiration rate and reduces the CO_2 availability for photosynthesis. However, limited mesophyll diffusion of CO_2 is observed (Flexas et al., 2007). To cope with the primary effects of salinity, plants regulate their osmotic potential and compartmentalise toxic ions. The regulation of osmotic potential to maintain turgor pressure (despite a lower water potential) involves several processes such as the uptake of K^+, the compartmentalisation of Na^+ and Cl^- into the vacuole, and the synthesis of compatible

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solutes such as proline, glycine betaine, polyol, sugars etc. (Ashraf, 1994). At the molecular level, the mechanisms involved in Na^+ compartmentalisation are well documented in *Arabidopsis thaliana* and many genes encoding Na^+ co-transporters and regulators (e.g., *NHX1*, *SOS1*, *SOS2* and *SOS3*) have been characterised (Apse et al., 1999; Guo et al., 2004; Shi et al., 2000). However, chloride uptake and the associated transporters are still poorly documented. Colmenero Flores et al. (2007) identified a chloride transporter presumably involved in long-distance transport and plant development. A secondary effect of toxic ions during salt stress (Gómez-Cadenas et al., 1996), along with other abiotic stresses such as drought (Chaves and Oliveira, 2004) and temperature (Sala, 1998), is the triggering of oxidative stress (Gueta-Dahan, 1997; Tanou et al., 2009), which causes damage to the leaf photosynthetic machinery (Allen and Ort, 2001). Like many other crops, citrus species are classified as salt sensitive (Maas, 1993). Citrus include three major genera which are sexually compatible: *Citrus*, *Poncirus* and *Fortunella*. Most of the rootstocks currently used belong to the *Citrus* and *Poncirus* genera, or are hybrids obtained from crosses of these two related genera (Barrett, 1985). The adverse effects of salinity on commonly used genotypes have been extensively reported in the literature (Anjum, 2008; Atmane et al., 2003; Garcia-Sanchez and Syvertsen, 2009; Lopez-Climent et al., 2008; Saleh et al., 2008) and include symptoms of leaf injury, growth suppression and yield decline. The primary effects of salinity in citrus are decreased stomatal conductance leading to reduced CO_2 diffusion and ultimately decreased net photosynthesis (García-Sánchez and Syvertsen, 2006) and increased ion accumulation (Brumós et al., 2010). Rangpur lime (*Citrus limonia* Osbeck), Sunki mandarin (*Citrus sunki* Hort. ex Tan.) and Cleopatra mandarin (*Citrus reshni* Hort. ex Tan.) are considered tolerant, while trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and its hybrids such as Carrizo citrange [*Citrus sinensis* (L.) Osbeck × *P. trifoliata* (L.) Raf.] are considered salt sensitive (Maas, 1993). Citrus damage caused by salinity is usually associated with chloride accumulation but not with sodium accumulation (Banuls et al., 1997; Moya et al., 2003). Trifoliate orange and its hybrids are considered poor Cl^- excluders (Cooper, 1961; Peynado and Young, 1969) although they have a great capacity to exclude sodium at low salinity levels (Walker, 1986). Citrus rootstocks such as Cleopatra mandarin and Rangpur lime are Cl^- excluders, thus explaining the salt-tolerant nature of these rootstocks (Cooper et al., 1952; Zekri and Parsons, 1992). Walker and Douglas (1983) observed significant differences between Rangpur lime, Kharna khatta (*Citrus karna*), and Etrog citron (*Citrus medica*) in the Cl^- accumulation in the leaves but little differences concerning the Na^+ accumulation. According to Moya et al. (2003) if Cl^- absorption is not limited at the root level, the ions will be translocated to the leaves via the transpiration stream and they will cause necrosis and even defoliation.

Both rootstocks and scions may influence the accumulation of Cl^- in the leaves (Banuls et al., 1990; Garcia-Legaz et al., 1993; Nieves et al., 1991). Scion effects may be apparent when rootstocks are poor excluders (Lloyd et al., 1989). Extensive analyses of the responses to salt stress in the currently used rootstocks have been conducted at the physiological and molecular level (Brumós et al., 2009). However, a global evaluation of the salt stress tolerance of genotypes commonly used as citrus rootstocks along with genotypes used as scions and other types with less commercial application, has never been reported. In this report, 12 citrus genotypes representing different *Citrus* species and the three *Citrus* genera were subjected to moderate salt stress to investigate and characterise their salt stress tolerance as well as their potential usefulness for breeding programmes.

2. Materials and methods

Twelve citrus genotypes representative of the diversity of Citrus *genera* were selected (Table 1). Plant materials were propagated by two methods: for polyembryonic genotypes, propagation was performed by sowing seeds in a neutral substrate (perlite); for monoembryonic species such as *C. medica* and *Citrus maxima*, stem cuttings were used to produce true-to-type plants. Rhizopon (auxin 4%, Rhizopon, Hazerswoude-Rijndijk, Netherlands) was used for root induction. Seedlings and rooted cuttings were transplanted 3 months after germination into 2.5-litre pots in a mixture of river sand and soil both from Corsica (classified as Cambisol with 15–25% clay, 34% silt and 42% sand) and peat (1:1:1). The plants were grown for 6 months in a greenhouse. Experiments were performed during the summer in a greenhouse under natural photoperiod conditions with a temperature regimen of 15–19 °C (night) and 24–29 °C (day), and a relative humidity between 60% and 85%.

2.1. Verification of the genetic conformity and ploidy status

The genetic constitution of the seedlings was analysed using four inter-simple sequence repeat (ISSR) primers: $HVH(CA)_7T$, $DBDA(CA)_7$, $BDB(CA)_7C$, $HVH(TCC)_5$ (Fang and Roose, 1997) (Table 2). PCR products were analysed using vertical denatured electrophoresis polyacrylamide gels (5% polyacrylamide, 7 M urea) or 1.5% agarose gels in 1X TBE buffer (45 mM Tris,

Table 1
List of citrus genotypes representing diversity, subjected to salt stress.

Common name	Tanaka system	ICVN [a] or SRA [b] no.	Code
Cleopatra mandarin	*C. reshni* Hort. Ex Tan.	SRA 948	CM
Sunki mandarin	*C. sunki* Hort. Ex Tan.	ICVN 0110076	SM
Australian sour orange	*Citrus aurantium* (L.)	SRA 851	ASO
Star ruby grapefruit	*C. paradisi* Macf.	SRA 293	SRG
Eureka lemon	*C. limon* (L.) Osb.	SRA 4	EL
Mexican lime	*C. aurantifolia*	SRA 140	ML
Combava	*C. hystrix* D.C.	SRA 630	CO
Engedi pummelo	*C. maxima* (Burm.) Merr	SRA 610	EP
Poncire commun citron	*C. medica*	SRA 601	PCC
Carrizo citrange	*C. sinensis* × *Poncirus trifoliata*	SRA 796	CC
Pomeroy poncirus	*Poncirus trifoliata* (L.) Raf	ICVN 0110278	PP
Marumi kumquat	*Fortunella japonica (Thunb.) Swing.*	SRA 488	MK

[a] International Citrus Variety Numbering.
[b] Agronomical Research Station Numbering.

Table 2
ISSR primers used for the identification of the zygotic genotypes among the different *Citrus* genotypes (Fang and Roose, 1997).

Name	Sequence	Hybridation T (°C)	Fragment length (bp)
ISSR 1	$HVH(CA)_7T$	55.5	300–1100
ISSR 4	DBDA(CA)7	55	200–1500
ISSR 5	$BDB(CA)_7C$	58	200–1000
ISSR 8	$HVH(TCC)_5$	55.5	450–1400

45 mM boric acid and 1 mM M EDTA, pH 8) and silver-stained (Beidler et al., 1982). The ploidy status of the plants was determined by flow cytometry using leaf samples (Froelicher et al., 2007).

2.2. Salt stress application

After 1 month of acclimation in a greenhouse, plants were assigned at random into two blocks. According to availability, three to six plants per genotype were assigned for salt treatment and three plants were assigned as control plants. Salt stress was applied from mid-May to the beginning of August, 2009 under natural photoperiod conditions, with night/day temperatures ranging from 18 °C to 38 °C, and a relative humidity between 50% and 70%. Salt-treated plants were watered three times a week with the nutrient solution (fertilizer 28-14-14, ref 205, Fertil, France) supplemented with 75 mM NaCl. This was done at the same hour at field capacity to ensure that the treatments were uniformly applied. The control plants were irrigated only with the nutrient solution. Two leaves per plant at same height and same developmental stage were tagged randomly. These two leaves were used for the periodic measurement of the studied parameters. In a few cases, especially at the end of the experiment, some of the leaves of the sensitive genotypes dropped. In these cases, neighbouring leaves were used for further measurements.

2.3. Plant stem diameters and leaf symptoms

Plant stem diameters were measured using an Electronic LCD Digital Vernier Caliper (Model no. EBC143 China). The diameter of the control and salt-treated plants was measured initially and after 80 days. Leaf symptoms were observed throughout the experiment and represented different physiological disorders, i.e., leaf necrosis, stem necrosis, leaf pointed depigmentation and green leaf fall down.

2.4. Gas exchange measurements

Net photosynthetic rate (A) and stomatal conductance (gs) were determined with a portable gas exchange fluorescence system (GFS-3000 Heinz Walz GmBH, Germany) using a photosynthetically active radiation photon flux of 1000 μmol m^{-2} s^{-1}. Measurements were performed in the morning (8 to 11 AM) to avoid high external temperatures and low humidity. Leaf temperature was 28±2 °C, leaf to air vapour pressure difference was 2.4±0.4 kPa, and ambient CO_2 concentration was 370±3 μmol mol^{-1} within the cuvette of the portable gas exchange fluorescence system.

2.5. Maximum quantum yield of PSII, leaf greenness and flavonoid content measurements

The maximum quantum yield of PSII $[(F_m - F_0)/F_m]$, leaf greenness and flavonoid content were measured each week. The maximum quantum yield of PSII was measured at night using a portable fluorometer (Hansatech Ltd., Kings Lynn., UK). Leaf greenness and leaf phenolic compounds, mainly represented by flavonoids, were measured in the morning (8 to 11 AM) using a SPAD chlorophyll metre (Minolta SPAD-502, Japan) and an optical sensor Dualex 3.3® start-up (Force-A©), respectively.

2.6. Sodium and chloride content

Control samples (one to three leaves per replication depending on availability) and salt-treated samples (one to three leaves per replication with at least three replications) were collected after 10, 30, 50, 60 and 80 days of salt stress. Sodium and chloride analyses were carried out according to Mouhaya et al. (2010). Sodium and chloride leaf contents were expressed in mg g^{-1} of dry mass.

2.7. Statistical analysis

Data are expressed as the mean value±S.E. SIGMASTAT from SPSS (Systat Software Inc., San Jose, CA, USA) was used to analyse the data. ANOVA tests were used to detect differences between the genotypes and the growing conditions at the probability level of $P=0.05$.

3. Results

3.1. Genetic conformity and plant ploidy status

Among the four ISSR markers tested, only ISSR1 presented a polymorphism among all studied genotypes. This permitted the verification of the genetic origin of all the plants. All of the seedlings presented an identical genetic profile to those of their respective mother trees. Also, a selection based on plant morphology was conducted to remove a few plants displaying specific morphological characters. These plants represented possible mutants or hybrids that were not detected via genetic analyses, although the second hypothesis is improbable. An analysis of the seedlings using flow cytometry did not reveal any spontaneous autotetraploid plants.

3.2. Plant growth and leaf symptoms

As expected, the control plants exhibited a higher increase in stem diameter compared to the plants subjected to 80 days of salt stress (Fig. 1). Under control conditions, the greatest increase in stem diameter was observed for Pomeroy poncirus (103.3%±5.6). Combava displayed the lowest increase (36.0%±10.3) in stem diameter. For salt-treated plants, Pomeroy poncirus presented the greatest increase (58.9%±3.3) while Marumi kumquat had the smallest increase of stem diameter (6.3%±2.5) (Fig. 1).

The appearance of leaf damage symptoms were noted during the salt stress experiment. Three types of leaf symptoms were

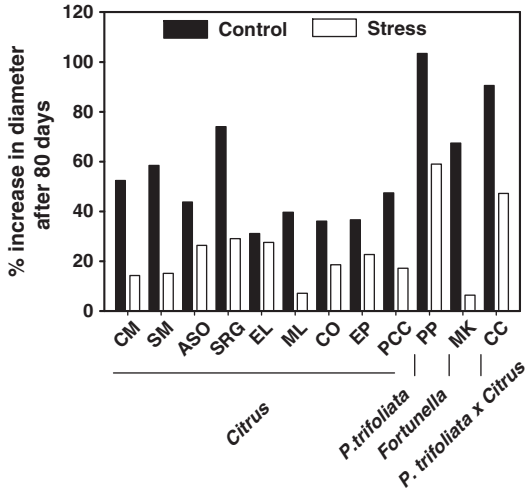

Fig. 1. Percentage increase in the stem diameter of control and stressed plants after 80 days of salt stress (75 mM NaCl) treatment. The black bar represents the controls and the white bar represents stressed plants ($n \geq 3$). Names of the genotypes are presented in Table 1.

observed: necrosis, yellowing and leaf fall down (Fig. 2). The most affected genotypes were Poncire commun citron and Carrizo citrange. In these genotypes, symptoms of necrosis occurred earlier than in the other genotypes. Leaf necrosis began from the border of the leaves and then spread to the central vein. In Carrizo citrange, the symptoms first appeared in the lower leaves and then progressively spread to the upper leaves. Star ruby grapefruit and Marumi kumquat showed sudden symptoms of leaf fall. However, the emergence of new leaves was observed only in Star ruby grapefruit. The yellowing of the leaves followed by the leaves falling was observed in Mexican lime. The leaves of Eureka lemon presented only yellowing while the appearance of small yellow dots was observed on the upper leaves of Pomeroy poncirus. Australian sour orange, Cleopatra mandarin, Sunki mandarin, Engedi pummelo, and Cambava did not show any leaf damage symptoms.

3.3. Leaf chloride and sodium content

Salt treatment led to increased leaf chloride and sodium concentrations in all of the tested genotypes. For clarity purposes,

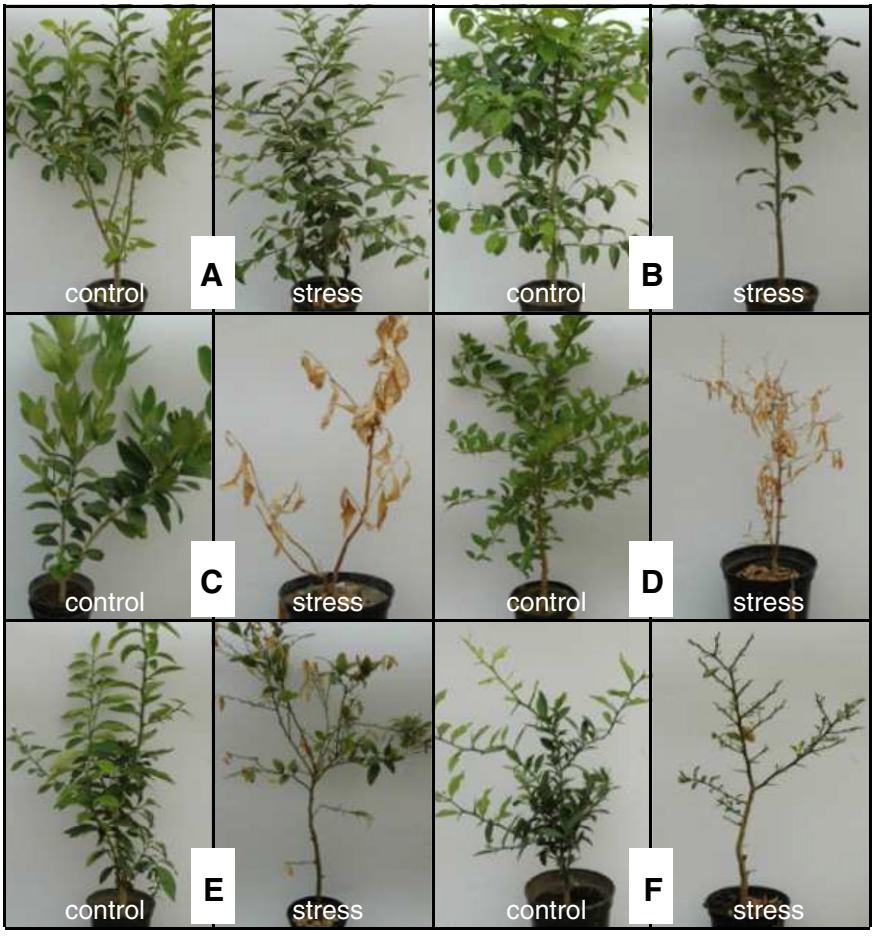

Fig. 2. Symptoms presented by genotypes during salt stress. (A) Lemon did not exhibit any symptoms after 80 days. (B) Australian sour orange presented a reduction of vegetative growth. (C) Citron presented leaf and branch necrosis. (D) Kumquat showed leaf drop and necrosis after only 45 days. (E) Mexican lime presented leaf drop while grapefruit (F) presented leaf drop and new leaf synthesis.

only a few genotypes representing the range of chloride and sodium contents were selected to be plotted on the graphs of Fig. 3A and C. The genotype choice was also made to be representative of the citrus diversity concerning the different phenotypic traits.

After 80 days of stress, Poncire commun citron accumulated the highest level of chloride (109.1±15.6 mg g^{-1} DW), while Cleopatra mandarin accumulated the lowest level (12.1±5.8 mg g^{-1} DW). For all of the genotypes investigated, leaf chloride concentrations for the control plants ranged from 1.0 to 2.1 mg g^{-1} DW. Therefore, the genotypes were ranked according to the value of the ratio of the leaf chloride contents after 80 days of stress compared to the leaf chloride content during control conditions (Fig. 3B). A wide range of accumulation was observed, and a group of low chloride-accumulating genotypes (Cleopatra mandarin to Combava) was clearly identified.

Salt treatment also increased the leaf sodium content in all of the genotypes as illustrated in Fig. 3D. The accumulation of sodium was lower compared to the chloride level in all of the genotypes. Poncire commun citron accumulated the highest level (40.9±10.5 mg g^{-1} DW), while Cleopatra mandarin (11.6±3.9) accumulated the lowest level. For all of the genotypes investigated, the leaf sodium content of the control plants ranged from 0.09 to 1.16 mg g^{-1} DW. The same ranking of the genotypes used in Fig. 3B is presented in Fig. 3D according to the leaf sodium content analysis. A wide range of leaf sodium accumulation was also observed among the genotypes and the ranking was quite similar to the one for chloride accumulation.

3.4. Leaf greenness and phenolic compound content

Illustrations of the changes in leaf greenness and phenolic compound content for some of the genotypes throughout the stress treatment are shown in Fig. 4A and C. Additionally, using the same genotype ranking used in Fig. 3B, the ratio of leaf greenness and leaf phenolic content of the treated plants was measured 77 days after the initiation of stress treatment. The values were reported relative to the value measured during control conditions (Fig. 4B and D, respectively). For all of the genotypes tested, the salt-treated plants showed a decrease in leaf greenness. However, a marked decrease was observed for Marumi kumquat only after 50 days of salt stress (Fig. 4A). After 80 days of salt stress, with the exception of Marumi kumquat, the acutest decrease in leaf greenness was observed for Carrizo citrange (60%), Pomeroy poncirus (50%), and Poncire commun citron (49%); Cleopatra mandarin and Sunki mandarin exhibited only small decreases in leaf greenness, 10% and 14%, respectively. For all of the genotypes, a clear increase in the

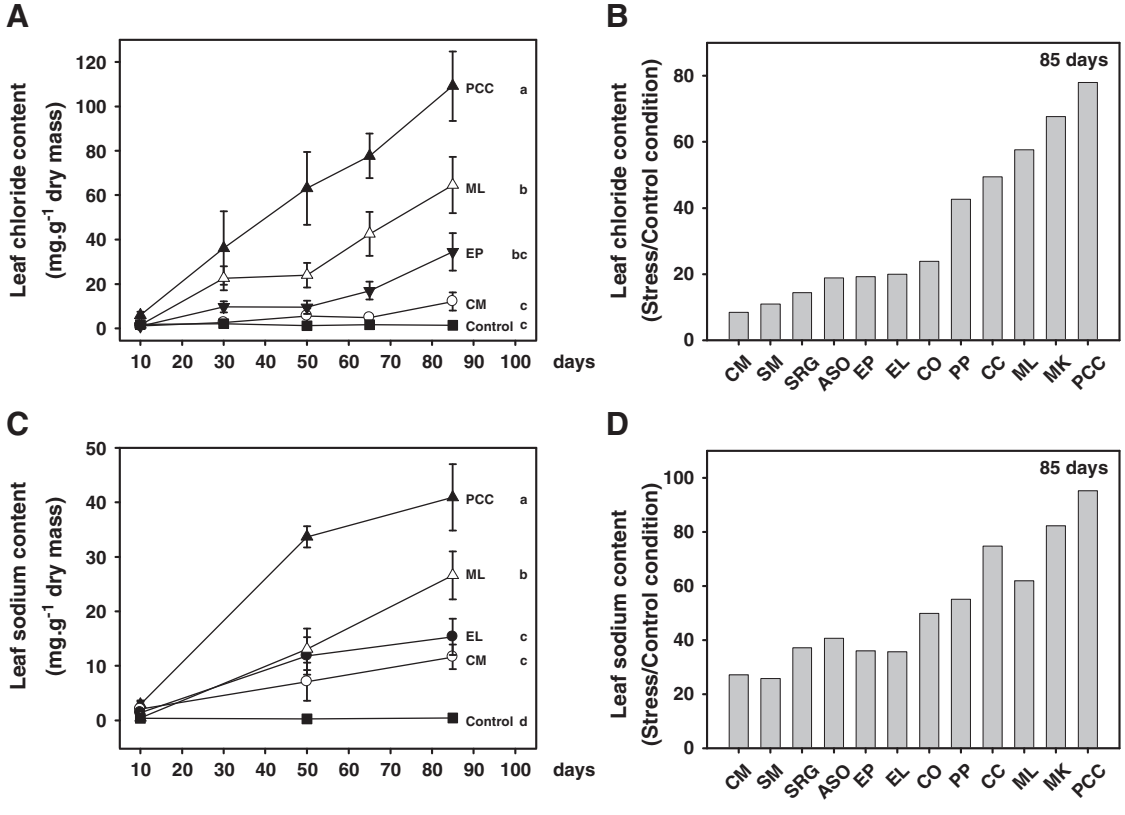

Fig. 3. (A) The leaf chloride content of four genotypes during 85 days of salt stress. For each date, the control represents the mean value of the 12 genotypes. (B) Leaf chloride content was based on the ratio of the leaf chloride content after 85 days of salt stress over the leaf chloride content measured in the control condition for the 12 investigated genotypes. (C) Leaf sodium content of four representative genotypes for 85 days. For each date, the control represents the mean value for the 12 genotypes. (D) The leaf sodium content indicates the ratio of the sodium content after 85 days of salt stress over the leaf sodium content measured under control conditions for the 12 investigated genotypes. Values (mean±S.E.) with different letters were significantly different ($P<0.05$, $n\geq3$). Names of the genotypes are presented in Table 1.

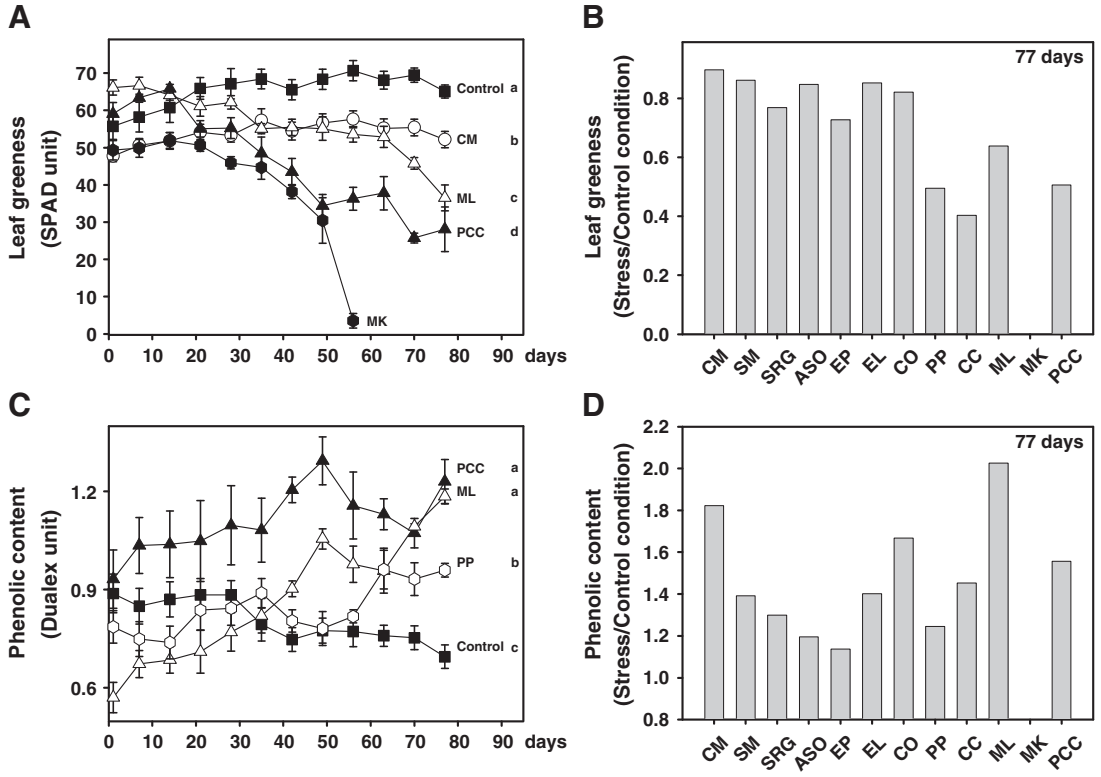

Fig. 4. (A) Leaf greenness of four representative genotypes for 77 days. For each date, the control represents the mean value for the 12 genotypes. (B) Leaf greenness was based on the ratio of the leaf greenness after 77 days of salt stress over the leaf greenness measured under control conditions for 11 genotypes. (C) Leaf phenolic content of three repetitive genotypes for 77 days. For each date, the control represents the mean value for the 12 genotypes. (D) Leaf phenolic content based on the ratio of the phenolic content after 85 days of stress over the phenolic content measured during control conditions for 11 genotypes. Values (mean ± S.E.) with different letters were significantly different ($P<0.05$, $n \geq 3$). MK is not represented on graphs B and D because this genotype was not healthy after 60 days of stress. Names of the genotypes are presented in Table 1.

phenolic compounds within the leaves was observed during the stress; the highest increase was observed for Mexican lime and the lowest was observed for Eingedi pummelo (Fig. 4D).

3.5. Maximum quantum yield of PSII and gas exchange measurements

An illustration of the changes of $(F_m - F_0)/F_m$, *gs* and *A* in representative genotypes is presented in Fig. 5A, C and E. The ratios represent the values measured during the stressed condition over the control condition for $(F_m - F_0)/F_m$, *gs* and *A* after respectively 60, 80 and 80 days using the same genotype ranking used in Fig. 2B (Fig. 5B, D and F). Marumi kumquat exhibited the highest decrease in $(F_m - F_0)/F_m$ (35%) after only 43 days of stress. Three other genotypes, Carrizo citrange, Mexican lime, and Poncire commun citron, also showed drops in $(F_m - F_0)/F_m$ (24%, 19% and 16%, respectively). Interestingly, Pomeroy poncirus presented only a small decrease in $(F_m - F_0)/F_m$ (3%), while genotypes such as Star ruby grapefruit, Cleopatra mandarin, Sunki mandarin, Eingedi pummelo, and Eureka lemon did not show any significant change. Gas exchange measurements were recorded for all of the genotypes with the exception of Pomeroy poncirus and Marumi kumquat because the leaf size of these genotypes was too small. Stomatal conductance decreased after 10 days of salt treatment for nearly all genotypes. The most significant decrease in *gs* was observed in Combava and Mexican lime, while Australian sour orange, Carrizo citrange and Cleopatra mandarin were less affected (Fig. 5D). The results regarding stomatal conductance and net photosynthetic rate showed the same pattern for all of the genotypes studied. Interestingly, Eureka lemon and Combava maintained a photosynthetic rate similar to Sunki mandarin, Star Ruby grapefruit and Poncire commun citron (Fig. 5F).

4. Discussion

Most of the citrus genotypes that have been characterised for abiotic tolerance are rootstocks belonging to the *Citrus* and *Poncirus* genera, or are hybrids obtained from crosses made between these two related genera. However, little is known about genotypes belonging to the *Fortunella* genera or monoembryonic citrus genotypes such as citron or pummelo. These genotypes may present a high diversity of responses with potential genotypes well adapted to salt stress. Therefore, sexual or somatic hybridisation may permit the recovery of apomixis and may enable the production of new rootstocks better adapted to salt stress.

To increase the genetic gain through breeding, a wide range of citrus genotypes covering the available diversity was investigated. This investigation hoped to identify different traits of sensitivity/tolerance that were supported by specific physiological determinants. Two amplification methods were used for the experimental plant materials: cuttings were used for monoembryonic varieties

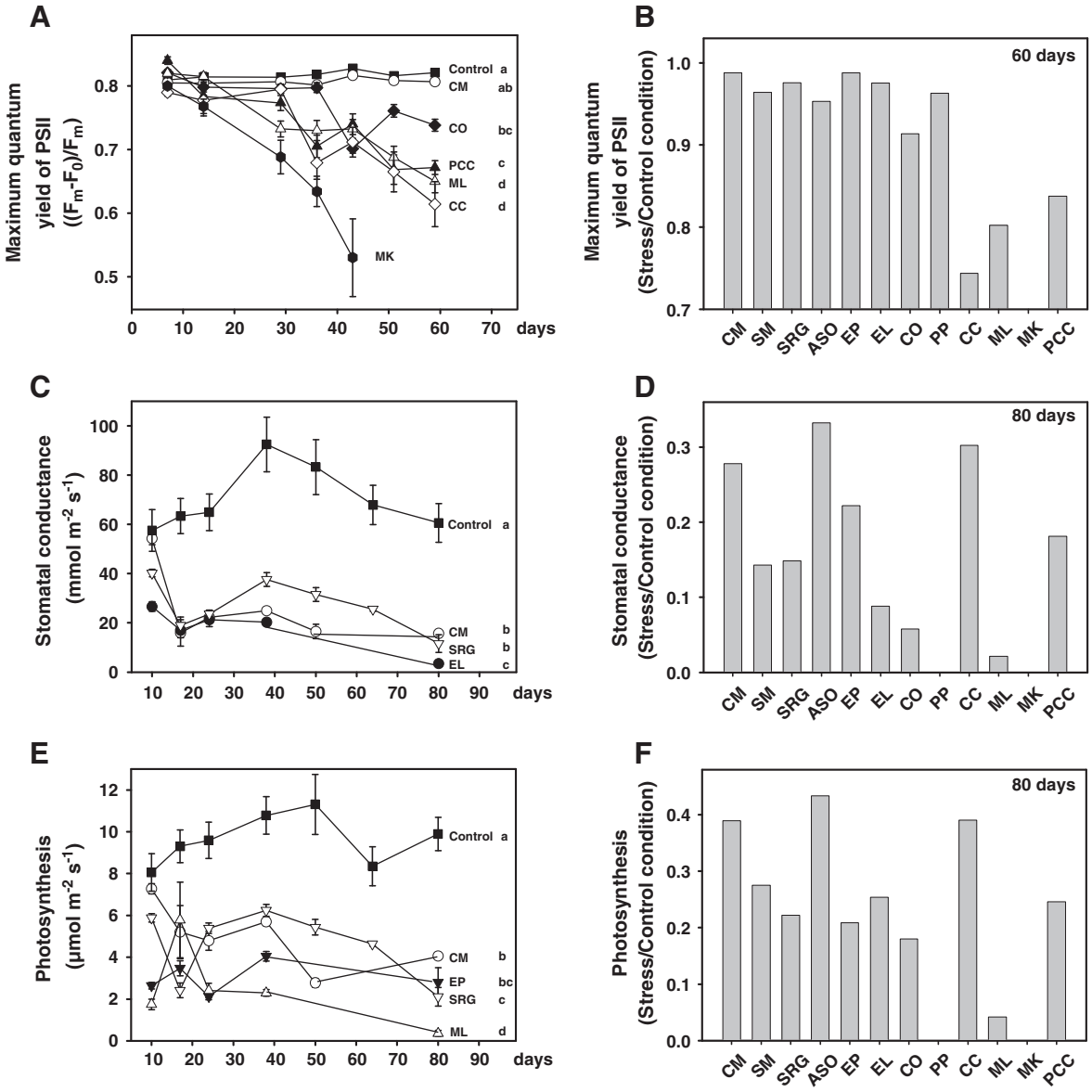

Fig. 5. (A) Maximum quantum yield of PSII of six repetitive genotypes during 60 days of salt stress. For each date, the control represents the mean value for the 12 genotypes. (B) Maximum quantum yield of PSII based on the ratio of the maximum quantum yield of PSII after 60 days of salt stress over the maximum quantum yield of PSII during control conditions for 11 genotypes. (C) Stomatal conductance of three repetitive genotypes during 80 days of salt stress. For each date, the control represents the mean value for 10 genotypes. (D) Stomatal conductance was based on the ratio of the stomatal conductance after 80 days of stress over the stomatal conductance measured under control conditions for 10 genotypes. (E) The photosynthetic rate (µmol m^{-2} s^{-1}) of four repetitive genotypes during 80 days of salt stress. For each date, the control represents the mean value for 10 genotypes. (F) Photosynthetic rate was based on the ratio of the photosynthetic rate after 80 days of stress over the photosynthetic rate measured during control conditions for 10 genotypes. Values (mean±S.E.) with different letters were significantly different ($P<0.05$, $n\geq3$). MK is not represented on graph B because this genotype was badly injured after 60 days of stress. PP and MK were not represented on graphs D and F because the size of their leaves was too small for measurements of photosynthetic rate and stomatal conductance. Names of the genotypes are presented in Table 1.

and seedlings were used for polyembryonic varieties. Cuttings are not equally effective for all citrus varieties. Thus, the seedling approach was preferred for the polyembryonic varieties. The amplification method does affect some physiological characteristics of the tree. Multiplication from juvenile tissues (seeds) causes the expression of juvenile characters such as the presence of thorns. The amplification mode also determines root architecture. Stem cuttings taken from juvenile plants develop new roots more readily than cuttings taken from mature plants (Ferguson and Young, 1985). Furthermore, cuttings from young seedlings tend to produce roots that grow downward. Cuttings from mature trees tend to produce roots that grow laterally (Johnston et al., 1959). In this experiment, the plants were grown for 6 months prior to salt stress treatments. This growth delay ensured that the root volume was the same for all of the varieties, regardless of the propagation method. This was verified at the end of the experiment, when the roots of all the varieties were shown to occupy the entire pot. Thus, we can consider that the application of salt stress was relatively homogeneous for all of the varieties. The various measured parameters were analysed relative to the control plants to minimise

any potential effects of heterogeneity due to the physiological mode of amplification.

Several physiological parameters were studied to assess the variability of behaviour during a 12-week moderate salt stress treatment (75 mM). Uniformly-sized seedlings of the selected genotypes were investigated using SSR markers to remove potential zygotic plants since such genotypes may skew the response to salt stress.

4.1. Salt stress tolerance of highly sensitive and highly tolerant rootstocks used in the citrus industry

Physiological responses of citrus to alleviate salt stress are related to the plant's ability to restrict Cl^- transport from the root to the shoot (Iglesias et al., 2007; Moya et al., 2002, 2003; Romero-Aranda et al., 1998; Storey and Walker, 1998). Indeed, ion accumulation in the leaf significantly impacts different critical processes that are essential for plant survival, including *A*, *gs* and Fv/Fm. Correlations between the decrease in CO_2 assimilation and high Cl^- and Na^+ contents in leaves were previously reported in citrus genotypes (Anjum, 2008; Garcia-Legaz et al., 1993; Walker and Douglas, 1983).

After 12 weeks of salt stress, Pomeroy poncirus and Carrizo citrange, species known to be among the most salt sensitive rootstocks, were in the middle range of the Cl^- and Na^+ accumulating genotypes and presented higher growth rates as illustrated by the percentage increase in plant stem diameter (Fig. 1). During salt treatment, a high stress/control ratio for *gs* and *A* was observed for Carrizo citrange (Fig. 5D and F). Recently, Brumos et al. (2009) showed that Carrizo citrange had a high growth rate associated with higher photosynthetic activity and higher stomatal conductance. It was concluded that this phenotypic trait of Carrizo citrange, and probably also of Poncirus, was due to the overexpression of genes related to carbon metabolism and energy under salt stress, thus leading to the maintained growth rate associated with higher salt stress susceptibility. It is interesting to note that Cleopatra mandarin (known as one of the most tolerant citrus rootstocks), Sunki mandarin and Star ruby grapefruit presented the highest decrease in growth during stress treatments, as compared to control plants (Fig. 1). Under the stress conditions of this study, most of the genotypes quickly reduced their stomatal conductance (Fig. 5C) and photosynthetic activity (Fig. 5E) even though a wide range of Cl^- and Na^+ accumulation was observed (Fig. 3B, D). In the present study, Cleopatra mandarin was the lowest Cl^- and Na^+ accumulating genotype (Fig. 3A and C). Cleopatra mandarin and Australian sour orange (considered to be the second most tolerant citrus rootstock used in the citrus industry) both exhibited high CO_2 assimilation that was correlated with low chloride accumulation. However, Australian sour orange maintained $(F_m-F_0)/F_m$, *gs* and *A* values greater than other genotypes that have not been previously analysed for stress tolerance which may explain for part why this genotype is more sensitive to salt stress than Cleopatra mandarin. Indeed, an important defensive mechanism reported in Cleopatra mandarin, in response to salinity (Brumos et al., 2009), was decreased photosynthesis and stomatal conductance. PSII was not damaged, and this was associated with the repression of central metabolic processes such as carbon utilisation. In the end, these mechanisms may help the plant cope with oxidative stress (Tanou et al., 2009).

4.2. Salt stress tolerance in citrus varieties

Little is known about the salt stress tolerance mechanisms of many citrus genotypes typically used as commercial cultivars. In Poncire commun citron, salt stress induced leaf yellowing, necrosis, apparition of dots on the leaves, and ultimately, leaf fall. Additionally, a large decrease in the growth rate (Fig. 1) was associated with a limited decrease in *gs* and *A* (Figs. 5D and 3F) despite observations of high Cl^- and Na^+ accumulation (Fig. 3B and D). This suggests that photosynthesis and PS II were primarily unaltered. This may implicate other mechanisms besides toxic ion avoidance as involved in salt tolerance. This may include intra-cellular compartmentalisation (Neumann et al., 1997) or increased oxygen radical-scavenging ability (Arbona et al., 2003; Yasar et al., 2006).

Eureka lemon is a hybrid of Australian sour orange and citron (Nicolosi et al., 2000). The results of this study suggest that the salt sensitivity of citron was not transmitted to Eureka lemon. Eureka lemon accumulated fewer toxic ions and exhibited lower *A* and *gs* values. A lower value of $(F_m-F_0)/F_m$ is usually a direct effect of oxidative stress and is typically correlated with a great decrease in *A* and *gs* conductance (Hernandez et al., 2000; Mouhaya et al., 2010). Interestingly, $(F_m-F_0)/F_m$ values were little changed for most of the genotypes (Fig. 5B), suggesting that PSII was not affected. In Eureka lemon, *gs* and *A* were among the lower values measured after 80 days of stress (Fig. 5C, F). For this genotype, the percentage increase in diameter was very similar under control and salt stress conditions (Fig. 1). This suggests that Eureka lemon may decrease its metabolism to a greater extent than Cleopatra mandarin to cope with salt stress (Brumos et al., 2009).

Other genotypes such as Mexican lime and Marumi kumquat showed a large decrease in the growth rate associated with a large decrease in $(F_m-F_0)/F_m$. The same leaf symptoms of Poncire commun citron were observed in Mexican lime. Lime is parented with citron (Nicolosi et al., 2000). However, in Marumi kumquat, very early and pronounced symptoms of discolouration and defoliation (Fig. 2) without leaf burn, typically associated with early death, were observed.

Although Marumi kumquat belongs to another genus (*Fortunella*), it responded to salt stress in a manner similar to Mexican lime. Marumi kumquat exhibited a high leaf chloride content and leaf drop. This suggests that the high salt sensitivity of these genotypes was due to high Cl^- and Na^+ accumulation in the leaves, assuming that no exclusion of this toxic ion occurred at the root level as has been reported in Cleopatra mandarin (Cooper et al., 1952; Zekri and Parsons, 1992). It is hypothesised that intra-cellular compartmentalisation and oxygen radical-scavenging ability may be very limited in these genotypes.

Pummelos belong to the *C. maxima* species and are one of the putative parents of sweet orange (*C. sinensis*) and grapefruit (*Citrus paradisi*) (Nicolosi et al., 2000). Based on this study, these genotypes are quite tolerant to salt stress. Eingedi pummelo accumulated less Cl^- and Na^+ than other salt sensitive genotypes. In Eingedi pummel, $(F_m-F_0)/F_m$ values were little altered. This

suggests that PSII system was not damaged, but instead gas exchange and chlorophyll content were maintained. Despite being monoembryonic, pummelo seedlings are considered well adapted to abiotic constraints such as high pH or calcareous soil. Pummelo seedlings have also been selected along with mandarin genotypes to generate somatic hybrids tolerant to biotic and abiotic traits (Grosser et al., 2004). Therefore, using pummelo in classical breeding programmes and somatic hybridisation breeding programmes may be promising for the production of new rootstocks better adapted to biotic and abiotic constraints.

Star ruby grapefruit presented sudden symptoms of leaf fall while leaves were still green. Subsequently, new leaves emerged. These characteristics during stress are very specific and likely related to specific molecular mechanisms since low leaf accumulation of Cl^- and Na^+ occurred even when leaf symptoms were observed for this genotype. Indeed, this behaviour could be characterised as a defence mechanism that involves an evasive action to eliminate leaves that have accumulated toxic ions and produce new leaves for the maintenance of photosynthesis and plant growth (Arbona et al., 2003, 2009; Iglesias et al., 2007). Under salt stress conditions, a wide range of plant nutritional deficiencies have been reported in citrus (Alva and Syvertsen, 1991), and leaf abscission reportedly contributes to salt-induced growth inhibition in citrus (Gómez-Cadenas et al., 1998). Additionally, growth regulation hormones such as ABA and ethylene have been identified as main modulators of leaf abscission (Gómez-Cadenas et al., 1998, 2003a, 2003b). However, it is assumed that besides Cl^- ion content, and ABA and ethylene hormonal balance, there are other phenomena responsible for leaf drop. These include 1-aminocyclopropane-1-carboxylic acid (ACC) content and specific hormonal ratios (Zeatin + Zeatin-riboside/ACC). These parameters have been correlated with the onset and progression of leaf senescence (Ghanem et al., 2008).

5. Conclusion

In conclusion, the ability of genotypes to tolerate salt stress is related to their capacity to reduce central metabolic processes related with carbon utilisation and toxic ion exclusion. Salt stress tolerance only appears to be present in the *Citrus* genus and mainly in two fundamental species, mandarins (*Citrus reticulata*) and pummelos (*C. maxima*). Species and cultivars such as Australian sour orange, lemon and grapefruit, whose genetic origin is linked to pummelo and mandarin, possess inherited forms of tolerance from their parents. Combava (*Citrus hystrix*), a member of wild *Citrus* species grouped within the subgenus Papeda, can be also qualified as tolerant. Thus, this study suggests expanding the investigation of salt tolerance for rootstock breeding programmes within *Citrus*, to mandarins, pummelos and Papedas.

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